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Embryonic Stem Cell Self-Renewal, Analyzed

Maintaining the pluripotency of mouse ES cells requires both LIF (leukemia inhibitory factor) and unknown factors in serum. The paper from Ying et al. in this issue of *Cell* shows that BMP (bone morphogenetic protein) can replace serum in this capacity, defining molecular requirements for ES cell self-renewal.

The inner cell mass (ICM), a cluster of pluripotent stem cells, each of which has the capacity to generate all types of embryonic tissues, exists only fleetingly during the blastocyst stage of mammalian development. Remarkably, the life of these cells can be extended in tissue culture, allowing production of embryonic stem (ES) cell lines, which in mouse have led to a new era of engineered mutants for experimental research, and in human to a new era of therapeutic exploration, at the same time engaging the world in intense ethical debate. Human ES cell lines have the potential to yield major advances in tissue repair, and their production and use are likely to become increasingly important areas of translational research. In this issue of *Cell*, Ying et al. (2003b) shed new light on the factors and pathways that stimulate mouse ES cell self-renewal, allowing, for the first time, ES cell line generation in a defined medium.

From the beginning, production of mouse ES cell lines was found to depend on addition of a mixture of exogenous factors in the culture medium, by growing with feeder cells and/or serum. But use of complex media clouds the critical question of which particular exogenous factors maintain self-renewal, promoting the artificial extension of ICM life. Self-renewal is the essential, defining characteristic of stem cells, and insight into the underlying mechanism in ES cells may well be pertinent to other stem cell types.

Previous studies revealed one of the essential self-renewal pathways for mouse ES cells: LIF and related cytokines that use the gp130 receptor, which work by activating the transcription factor STAT3 (Smith, 2001). However, these observations were made in serum-containing cultures, and without serum, LIF alone cannot maintain ES cell self-renewal; instead, the cells differentiate predominantly into neural phenotypes. Hence, there is another factor or factors needed in combination with LIF to achieve self-renewal. It is notoriously difficult to identify an active factor from serum—too many research years have been wasted fractionating serum samples. Instead, the authors pursued the observation that neural phenotypes develop without serum, leading to their idea that factors known to inhibit neurogenesis might be the key. So they began to look at BMP, a fundamental anti-neurogenesis factor in the early embryo. At the outset, it probably seemed rather unlikely that BMP would be the factor, because it can make ES cells differentiate into non-neural fates such as mesoderm and hematopoietic cells. However, the key experiment here was to test BMP in combination with LIF.

The authors show that when ES cells are grown in serum-free medium, BMP4 and LIF can indeed support ES cell line derivation and maintenance, even from single cells, which is the most rigorous test. GDF6 (growth and differentiation factor 6) also worked, but not TGF-β1 (Transforming growth factor β1), suggesting that within the TGF-β family of cytokines, the ability is shared by the BMP/GDF/MIS (Muellerian inhibiting substance) sub-family and not the other, TGF-β/Activin/Nodal. The resulting ES lines have normal karyotype, and are still pluripotent, because they can be injected back into embryos and contribute to chimeras, and they can be used to generate modified lines, similar to ES cells made by conventional methods. This discovery allows, for the first time, the production of ES cell lines in an entirely defined medium. There are great advantages to this new technique, as it will allow much more consistency in ES line production, and the ability to probe molecular aspects of ES biology without the confusion that comes from growing the cells in multiple, unknown, and variable factors.

The authors then explored the mechanism of BMP-LIF synergism. They established the existence in ES cells of transcripts for BMP4 and GDF6, implying the possibility of autocrine signaling, and for type I and type II serine-threonine receptors. Moreover, they demonstrated that BMP application induced rapid phosphorylation of Smad1, an essential step in BMP signaling. There are three obvious alternative synergistic pathways that could be operating in the ES cells: an enhancement of STAT3 signaling, an inhibition of the prodifferentiation/death signals Erk and p38 MAP kinases, and a complementary parallel pathway. In fact, the authors show that BMP has little effect on STAT3 signaling and causes a paradoxical stimulation of p38, confirming reports in other cell types (Iwasaki et al., 1999; Yamaguchi et al., 1999), and of Erk, as found previously in ES cells (Burdon et al., 1999), which were not inhibited in the presence of LIF; these data encouraged them to search for a parallel pathway.

Id (Inhibitor of differentiation) genes, which encode negative bHLH factors, can be induced by BMP in neuroepithelial cells and ES cells as a result of Smad activation. The authors introduced episomal Id1, Id2, and Id3 into ES cells and found that the resulting lines grew as well in LIF alone as parental lines did in LIF plus BMP. Moreover, exposure to serum increases Id expression in ES cells, so this pathway may explain the essential action of serum.

When the floxed Id construct was removed, lines without exogenous Id grown in LIF alone produced a high proportion of neurons. Surprisingly, the authors found that the neurogenic gene *Mash-1* was expressed in undifferentiated ES cells. Hence, they propose that Id functions to halt progression of neural differentiation triggered by precocious, or primed, expression of Mash-1 and other proneural bHLH factors, or non-bHLH partners involved in neural development such as Pax family mem-

bers. In addition, Ids can be direct positive regulators of cell growth by interacting with multiple cell cycle components (Norton, 2000).

Previous studies from this lab identified the transcription factor Nanog as vital for self-renewal, allowing Nanog-overexpressing ES cells to be maintained without LIF, although added LIF could still aid their self-renewal. They now find that cells overexpressing Nanog have no requirement for additional BMP, while LIF can still enhance their growth. This implies a strong connection between BMP signaling and Nanog activity, and in fact they found that Nanog sustains Id expression.

Another gem contained in this paper is the recognition that BMP signaling, like LIF signaling, has both stimulatory and inhibitory effects on self-renewal, leading the authors to propose that the balance of these may normally serve as a protection against runaway cell expansion. Thus, just as LIF signaling stimulates self-renewal via STAT3, and BMP signaling does so via Id, both also inhibit self-renewal by Erk and p38 activation. Overexpression of BMP, even in the presence of LIF, leads to differentiation into non-neural fates, further indicating that a balance of Smad and STAT signaling determines the choice between self-renewal and differentiation, and in this context it is interesting to note that expression of Socs3, a negative regulator of cytokine signaling, is enhanced by expression of both BMP and LIF compared to LIF alone. Exactly how the pro-differentiation versus pro-self-renewal forces are balanced is not clear, but it appears there are multiple interacting pathways operating via Smad and STAT independently, and also possibly via a Smad/STAT ternary complex.

In summary, the authors have shown that in defined media, ES cells make neural cells, and that self-renewal in ES cells is essentially an anti-neurogenic process, driven by the coordinate actions of BMP and STAT3 signaling pathways. These findings strengthen the idea that BMPs regulate the early choice between neuronal and non-neural fates in mammals, as in *Xenopus* (Tropepe et al., 2001), although in ES cells neural differentiation appears not to be a default state, but rather is FGF-dependent (Ying et al., 2003a). It seems, then, that even as early as the ICM stage, the pluripotent stem cell is balancing between maintaining self and taking on a neural alter ego, a process which (by Freudian coincidence) involves Id. Perhaps these revelations will allow more rapid pursuit of defined media for generating consistent human ES lines, for which the molecular basis of self-renewal still remains a mystery (Smith, 2001; Xu et al., 2002).

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Selected Reading

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Proteolytic Processing in Development and Leukemogenesis

There are now numerous examples in the hematopoietic system of genes that are critical for normal hematopoietic development, but when mutated, rearranged, or overexpressed, contribute to leukemogenesis. Two papers in this issue of *Cell* provide a fascinating twist on this paradigm, and suggest that proteolytic processing of certain of these genes plays an important role both in development and in leukemogenesis. These findings also suggest the possibility that proteases may be therapeutic targets in leukemia.

Hsieh et al. (2003a) have studied the role of proteolytic processing of the MLL gene product. MLL is a critical upstream effector of *HOX* gene expression in vertebrates, and is required for normal axial-skeletal transformation (Yu et al., 1998). As discussed in more detail below, MLL is also rearranged by chromosomal translocations in human leukemias, resulting in fusion of N-terminal *MLL* sequences with more that 40 different fusion partners of diverse function (Ayton and Cleary, 2001).

Hsieh et al. (2003a) identified a novel protease, Taspase 1, which cleaves MLL at two conserved D/GADD and D/GVDD sites, generating N-terminal 320 kDa (N320) and C-terminal 180 kDa (C180) fragments. Taspase 1 is an endopeptidase that utilizes an N-terminal threonine as the active site nucleophil to proteolyze polypeptide substrates following an aspartic acid residue. Taspase 1 is also a member of a diverse superfamily of N-terminal nucleophile hydrolases (Ntn-hydrolases) that are all activated as proenzymes by autocatalytic cleavage.

Taspase 1-mediated proteolytic cleavage of MLL has differential effects in regulation of *HOX* gene expression. For example, knockdown of Taspase 1 using siRNA impairs maintenance of expression of 3′ *HOX A* genes (*HOX A1*, *A3*, *A4*) that are expressed early, but not of late 5′ *HOX A* genes (*HOXA9, HOXA10*). In contrast, *MLL* knockdown or *Mll* null ES cells display more global defects in *Hox* gene expression.

These differential effects on *HOX* gene expression of complete loss of function of MLL versus loss of Taspase 1 suggest that proteolytic cleavage of MLL and related family members by Taspase 1 may provide additional